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Protein degradation in cat liver cells

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1. Body proteins in cats were prelabelled with [^{14}C]valine, and protein degradation was studied in isolated hepatocytes. 2. Amino acids appeared to have a direct inhibitory effect on protein degradation, but the effects were generally smaller than those previously shown in the rat. 3. The amino acid control of protein degradation in the cat differs from that in the rat, as shown by the lack of effects of glutamine, asparagine, arginine or methionine in cat hepatocytes. This may be related to the unique features of protein metabolism of this species. 4. NH_4Cl, leupeptin and amino acids, which suppress lysosomal protein degradation by different mechanisms, caused less than 30% inhibition of protein degradation when used at the optimum concentrations reported for the rat. The ability of the lysosomal system to respond to nutritional deprivation is apparently lower in the cat than in the rat.

INTRODUCTION

The cat has a comparatively high protein requirement, which could be partly due to the low efficiency of utilization of dietary protein (Greaves & Scott, 1960). Rogers *et al.* (1977) reported the lack of adaptation of enzymes regulating amino acid catabolism, gluconeogenesis and ureagenesis in cats fed on low- and high-protein diets. They reasoned that the high-protein requirement of the cat arises from its inability to change the activities of nitrogen-catabolizing enzymes, resulting in the high obligatory loss of nitrogen in the urine. However, we have shown that the cat can partially adapt to an increase in the amount of dietary protein, by increasing the flux rates of the catabolism of some amino acids in the liver (Silva & Mercer, 1985).

Since the cat is a carnivore, it is largely dependent on amino acids for the synthesis of glucose *de novo*. In the rat, the amount of amino nitrogen entering the liver from the gut is considerably less than that produced by the breakdown of body protein (Das & Waterlow, 1974), but to our knowledge there have not been any similar studies reported in the cat.

Liver protein turnover is increased by starvation and decreased by protein feeding, and the amount of protein in the liver is primarily controlled by changes in the rate of protein breakdown (Garlick *et al.*, 1973, 1975; Conde & Scornik, 1976). In the rat, protein degradation has been shown to be influenced by physiological compounds, e.g. amino acids and growth-promoting factors (Woodside & Mortimore, 1972; Warburton & Poole, 1977; Ballard *et al.*, 1980) and by non-physiological model compounds such as leupeptin (Dunn & Aronson, 1977; Bohley *et al.*, 1977; Hopgood *et al.*, 1977). However, little is known of the control of protein degradation in the cat. The experiments reported here were designed to see whether the high dietary protein requirement of the cat was reflected in the mechanisms of control of lysosomal protein degradation in hepatocytes.

MATERIALS AND METHODS

Chemicals

'Saffan' (alphaxalone and alphadolone acetate) was purchased from Glaxo (Australia), and insulin was from Nordisk, Gentofte, Denmark. Teric X 10 was obtained from I.C.I. (Australia) Ltd. L-[U-^{14}C]Valine (275 mCi/mmol) was purchased from Amersham (Australia). Collagenase was from Boehringer Mannheim (Australia). All amino acids and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., or were of analytical-reagent grade available from standard suppliers.

Amino acid mixtures

The mixture of essential amino acids was based on the medium described by Eagle (1959) and contained 2.5 mM-arginine, 0.63 mM-histidine, 1.6 mM-lysine, 2 mM-leucine, 2 mM-isoleucine, 0.5 mM-methionine, 1 mM-phenylalanine, 2 mM-threonine, 0.25 mM-tryptophan, 2 mM-valine, 1 mM-tyrosine and 0.5 mM-cysteine. The non-essential amino acid mixture was the same as that used by Hopgood *et al.* (1977) and contained alanine, asparagine, aspartic acid, glutamic acid, proline, serine and glycine in equimolar amounts (2.3 mM). The branched-chain amino acid mixture consisted of isoleucine, leucine and valine each at 5 mM. The mixture of phenylalanine, methionine and tryptophan contained 8, 4 and 2 mM respectively of the amino acids. The effects of single amino acids were tested at 10 mM, except for tryptophan, which was added at a concentration of 5 mM.

Preparation and incubation of hepatocytes

Cats (700–800 g) were fed with a commercial dry food *ad libitum*, and the body proteins were labelled 24 h before cell isolation by the injection of 100 μCi of L-[U-^{14}C]valine into the cephalic vein. Valine is only catabolized to a minor degree in the intact rat (Mortimore & Mondon, 1970), and a 24 h labelling period before isolation of cells has been shown to label the long-lived proteins, which are representative of general protein turnover (Seglen *et al.*, 1979). Hepatocytes were isolated from 12 h-starved cats by collagenase perfusion (Berry & Friend, 1969; Elliott & Pogson, 1977; Silva & Mercer, 1985). Cell incubations were conducted at 37 °C in complete Krebs–Henseleit (1932) buffer containing 1% defatted bovine serum albumin and enriched with 20 mM-lactate/pyruvate (9:1 molar ratio). After a 30 min preincubation period, the experimental

additions were made as iso-osmotic solutions. The stoppered incubation vessels were gassed with O_2/CO_2 (19:1) before and during each hour of an experiment. Incubations were terminated after 3 h by the addition of $HClO_4$ (final concn. 5%, w/v).

Indices of cellular integrity

The release of ^{14}C-labelled protein and lactate dehydrogenase (EC 1.1.1.27) into the medium was measured as indices of cell damage. Cell viability was estimated by Trypan Blue exclusion, and these parameters were determined during each hour of the incubation. The release of ^{14}C-labelled protein and that of LDH were determined after centrifuging (50 *g*, 2 min) 2 ml of cell suspension to separate the cells from the medium. Acid-soluble and acid-insoluble radioactivity (Hopgood *et al.*, 1977) and lactate dehydrogenase (Bergmeyer & Bernt, 1974) were measured in both fractions.

Measurement of protein degradation

All protein-degradation experiments were conducted in the presence of 2 mM-valine. All incubations and appropriate blanks were done in triplicate. After termination of incubations with $HClO_4$, the suspensions were kept at 4 °C for 30 min, then centrifuged at 1000 *g* for 15 min and the supernatants retained. The pellets were washed with 5% $HClO_4$, centrifuged (1000 *g*, 15 min) and the two supernatants combined. Then 25 μl of bovine serum albumin solution (40 mg/ml) was added to the supernatant, which was mixed thoroughly and centrifuged (1000 *g*, 15 min) and the supernatant assayed for acid-soluble radioactivity. Protein radioactivity was measured after dissolving the pellet in 750 μl of 2 M-NaOH. Radioactivity was measured in a 2,5-diphenyloxazole/1,4-bis-(5-phenyloxazol-2-yl)benzene/toluene/Teric X 10 scintillation mixture, with an LKB 1215 Rackbeta II liquid-scintillation spectrometer.

Statistical treatment of results

Percentage degradation of protein was calculated as described by Hopgood *et al.* (1977), and the results were treated as a randomized complete block in the analysis of variance. Treatment means in the Tables are presented with their standard errors (S.E.). Tests for statistical significance of the difference between the treatments and the control were conducted by using the S.E.M. obtained from the analysis of variance.

RESULTS

Hepatocyte integrity

The extent of the release of ^{14}C-labelled protein and lactate dehydrogenase (Table 1) was respectively lower than and similar to values reported from the same kind of experiments with rat hepatocytes (Hopgood *et al.*, 1977). Trypan Blue uptake increased by 5% during the 3 h incubation period.

Effect of amino acids on protein degradation

The rate of protein degradation was partially inhibited by the addition of several amino acids at 10 mM (Table 2). Tryptophan caused the greatest decrease (15.6%), followed by phenylalanine, leucine and isoleucine. Glutamine and asparagine did not inhibit degradation, and may have stimulated it.

The effect of various amino acid mixtures is shown in Table 3. The largest decrease was caused by the mixture of essential amino acids (22%) and by the phenylalanine/methionine/tryptophan mixture (16%). The addition of insulin to the essential amino acids and the non-essential amino acids caused a further decrease in the degradation rate. The mixtures of non-essential amino acids and of branched-chain amino acids caused a small but significant ($P < 0.05$) decrease in degradation. The transamination products of the branched-chain amino acids, i.e. 4-methyl-2-oxopentanoate, 3-methyl-2-oxopentanoate and 3-methyl-2-oxobutyrate (each at 2 mM), had no significant effects. The inhibitory effect of the mixture of essential amino acids was not affected by the addition of 5 mM-amino-oxyacetate.

Table 1. Integrity of cat hepatocytes during incubation for 3 h

Percentage release into the medium of lactate dehydrogenase and ^{14}C-labelled protein was calculated as 100 × activity in the medium/total activity. Values are means ± S.E. for the numbers of cats in parentheses.

	Release (%)	
Time...	0 h	3 h
Lactate dehydrogenase	5.1 ± 1.3 (4)	10.5 ± 1.8 (4)
^{14}C-labelled protein	1.7 ± 0.8 (5)	3.9 ± 1.2 (5)

Table 2. Inhibition of protein degradation by single amino acids

Cat hepatocytes prelabelled *in vivo* for 24 h by the administration of [U-^{14}C]valine were incubated for 3 h. All amino acids were added to give a final concentration of 10 mM, except tryptophan, which was added at 5 mM. Values are the means ± S.E. for five cats. The S.E.M. from the analysis of variance was 0.15, and significant difference ($P < 0.05$) from the control is indicated by an asterisk.

Amino acid	Protein degradation (%/3 h)
None (control)	7.12 ± 0.15
Arginine	7.02 ± 0.22
Asparagine	7.52 ± 0.20
Glutamine	7.43 ± 0.24
Histidine	6.81 ± 0.22
Isoleucine	6.39 ± 0.16*
Leucine	6.27 ± 0.16*
Methionine	7.28 ± 0.14
Phenylalanine	6.24 ± 0.13*
Proline	7.32 ± 0.21
Taurine	7.44 ± 0.29
Tryptophan	6.17 ± 0.15*

Reutilization of [^{14}C]valine derived from protein degradation and the effect of inhibitors

Incubations in the absence of valine caused a marked decrease in the apparent degradation rate, owing to a significant re-incorporation of degradation-derived [^{14}C]valine into protein (Table 4). Cycloheximide (10 μM), which prevents re-incorporation of [^{14}C]valine by inhibiting protein synthesis (Schreiber & Schreiber, 1973), caused only a partial inhibition of the apparent protein-degradation rate in the absence of valine.

Table 3. Effect of amino acid mixtures on protein degradation

The details of the amino acid mixtures and the incubation conditions are described in the Materials and methods section. Values are means ± S.E. for five cats, and the S.E.M. from the analysis of variance was 0.16, a significant difference ($P < 0.05$) from the control being indicated by an asterisk.

Addition	Protein degradation (%/3 h)
None (control)	7.12±0.15
Essential amino acids (16 mM)	5.53±0.23*
Insulin (0.15 nM)	6.70±0.22
Essential amino acids + insulin	5.24±0.26*
Non-essential amino acids (16 mM)	6.59±0.22*
Non-essential amino acids + insulin	5.98±0.14*
All amino acids (16 mM)	5.73±0.13*
Phenylalanine (8 mM) + methionine (4 mM) + tryptophan (2 mM)	6.00±0.28*
Essential amino acids − (Phe + Met + Trp)	6.48±0.14*
Leucine (5 mM) + isoleucine (5 mM) + valine (5 mM)	6.61±0.18*

Table 4. Effect of proteolysis inhibitors and the re-incorporation of [^{14}C]valine on protein degradation

Protein degradation was determined in the presence of 2 mM-valine, except where otherwise indicated. Values are means ± S.E. for five cats, and the S.E.M. from the analysis of variance was 0.13, a significant difference ($P < 0.05$) from the control being indicated by an asterisk.

Addition	Protein degradation (%/3 h)
None (control)	7.12±0.15
Leupeptin (0.25 mM)	5.22±0.21*
NH_4Cl (10 mM)	5.64±0.14*
Cycloheximide (10 μM)	6.76±0.18
No valine	5.56±0.18*
No valine + cycloheximide (10 μM)	6.20±0.19*

Leupeptin (0.25 mM), which inhibits lysosomal protein degradation by 70% in rat hepatocytes (Seglen *et al.*, 1979), caused an inhibition of only 30% in cat hepatocytes. The inhibition by NH_4Cl (10 mM) was also much lower than has been shown in the rat.

DISCUSSION

Several studies using rat liver preparations have shown that amino acids inhibit intracellular protein degradation. Investigators have used perfused livers (Mortimore & Mondon, 1970; Woodside & Mortimore, 1972) and isolated liver cells either in suspension (Hopgood *et al.*, 1977; Seglen *et al.*, 1980) or in monolayer culture (Sommercorn & Swick, 1981) to demonstrate the effects of different amino acids on lysosomal protein degradation. Our data shows that amino acids inhibit protein degradation in cat liver cells (Tables 1 and 2), but there are obvious differences from the rat.

Asparagine and glutamine, which are potent inhibitors of protein degradation in the rat (Seglen *et al.*, 1980), had no inhibitory effect when used at 10 mM in cat liver cells. Similarly, methionine and arginine, which also inhibited protein degradation in rat hepatocytes, albeit to a lesser extent (Hopgood *et al.*, 1977; Seglen *et al.*, 1980), had no effect in cat hepatocytes. A decrease in protein degradation by single amino acids has been demonstrated in rat liver cells in suspension (Hopgood *et al.*, 1977; Seglen *et al.*, 1980), but not in monolayer culture (Sommercorn & Swick, 1981). In our study, degradation was inhibited by a number of amino acids, but the magnitudes of the effects were less than has been shown in rat hepatocytes.

The effects of various amino acid mixtures on protein degradation in cat liver cells reported here were of a pattern similar to that observed in rat liver cells in suspension (Hopgood *et al.*, 1977) and in monolayer culture (Sommercorn & Swick, 1981). The mixture of phenylalanine, methionine and tryptophan inhibited protein degradation by 16%, accounting for 74% of the total inhibition seen with the mixture of essential amino acids. However, methionine by itself had no effect. Mortimore & Schworer (1977), using the perfused rat liver, showed that amino acids act directly by inhibiting the formation of autophagic vacuoles. However, in muscle, leucine must be transaminated to 4-methyl-2-oxopentanoate before it has an effect (Tischler & Fagan, 1982). Branched-chain amino acid transamination is primarily a function of muscle, but the oxidation of transamination products occurs mainly in the liver (Wohlhueter & Harper, 1970; Shinnick & Harper, 1976). Our results with the branched-chain amino acids and their transamination products show that the amino acids act directly on protein degradation in cat liver in a manner similar to that known to exist in the rat.

Cycloheximide inhibits both protein synthesis and degradation in many systems, including rat liver (Goldberg & St. John, 1976; Woodside, 1976; Ballard, 1977). In our experiments, cyclohexamide alone had no effect on protein degradation, and its function as an inhibitor of protein synthesis was less than expected, as it could not replace valine in its role to prevent re-incorporation of label. Although the reasons for this are unclear, it may reflect a lower potency of cycloheximide in the cat than in the rat, or it may point to different control mechanisms of protein metabolism in the cat.

Both leupeptin and NH_4Cl have been shown to inhibit protein degradation by 70% in hepatocytes prepared from 16 h-starved rats (Seglen *et al.*, 1979). Leupeptin inhibits cathepsins B, H and L, which are lysosomal proteinases (Kirschke *et al.*, 1976, 1977), but it may also inhibit extralysosomal proteinases in the liver cell, as it has been shown to inhibit calpain I in synaptosomal plasma membranes from rat brain (Siman *et al.*, 1983). The effect of NH_4Cl occurs as a result of an increase in lysosomal pH (Seglen & Reith, 1976; Carpenter & Cohen, 1976; Ascoli & Puett, 1978). The small inhibitory effect observed with NH_4Cl and leupeptin (less than 30%) in cat liver cells, when used at the maximum inhibitory concentrations reported for the rat, may point to a smaller contribution of the lysosomal component to overall protein degradation in the cat. Furthermore, amino acids which inhibit protein degradation by decreasing autophagic-vacuole formation (Mortimore & Schworer, 1977; Neely *et al.*, 1977) also effected less than 30% inhibition in cat hepatocytes.

Further evidence pointing to a lower lysosomal component in the cat can be obtained from a comparison of the rates of protein degradation in rat and cat hepatocytes incubated under nutritional 'step-down' conditions. Transferring rat liver cells from complete medium to one without serum and amino acids increases protein degradation from 1%/h to 4–5%/h, which is due mainly to an increase in lysosomal degradation (Mitchener *et al.*, 1976; Amenta *et al.*, 1977, 1978*a*,*b*; Seglen *et al.*, 1979). However, in cat liver cells incubated under 'step-down' conditions, the rate of protein breakdown was only 2.4%/h. In subsequent experiments not reported here, we have found the basal rate of degradation in cells in monolayer culture to be about 1.7%/h in the cat.

Although our results point to a higher non-lysosomal component of protein degradation in the cat liver, the lower response to nutritional 'step-down' conditions may also reflect constitutive differences in the lysosomal regulation of cellular protein balance between the cat and the rat. The evidence for non-lysosomal degradation of proteins is basically indirect (Ballard & Gunn, 1982), but it is well known to be unresponsive to physiological regulators and is generally believed to be involved in the degradation of short-lived proteins (Poole & Wibo, 1973; Epstein *et al.*, 1975; Knowles & Ballard, 1976; Neff *et al.*, 1979). Short-lived proteins comprise only a small fraction (less than 0.2%) of intracellular proteins (Hutson & Mortimore, 1980; Schworer *et al.*, 1981), but, owing to their rapid turnover rates, play an important role in the overall synthesis and degradation of protein in the steady state (Scornik & Botbol, 1976; Hutson & Mortimore, 1980).

Under physiological conditions, the amount of amino acids derived from protein degradation is quantitatively more important than the amino acids entering the liver from the gastrointestinal tract (Das & Waterlow, 1974). Amino acids in the liver are substrates for the opposing pathways of oxidation and protein synthesis, and cannot be reutilized with an efficiency of 100% for protein synthesis. The higher non-lysosomal component of protein degradation in the cat liver, which is not under physiological regulation, would continuously expose the amino acids derived from protein breakdown to competition from other amino acid-utilizing pathways. It is attractive to postulate that the amino acid requirements for protein synthesis therefore have to be met by a high dietary intake of protein by the cat.

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